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Enzymatic Formation of Unnatural Novel Polyketides from Alternate Starter and Nonphysiological Extension Substrate by Chalcone Synthase

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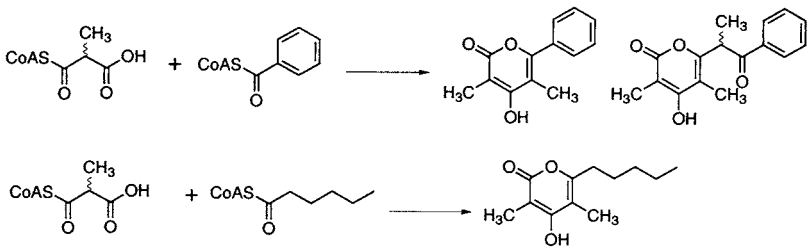
ABSTRACT



In the chalcone synthase (CHS) enzyme reaction, both the starter molecule and the extension unit of the poyketide chain elongation reaction were simultaneously replaced with nonphysiological substrates. When incubated with benzoyl-CoA and methylmalonyl-CoA as substrates, recombinant CHS from *Scutellaria baicalensis* afforded an unnatural novel triketide, 4-hydroxy-3,5-dimethyl-6-phenyl-pyran-2-one, along with a tetraketide, 4-hydroxy-3,5-dimethyl-6-(1-methyl-2-oxo-2-phenyl-ethyl)-pyran-2-one. On the other hand, the enzyme also accepted hexanoyl-CoA and methylmalonyl-CoA as substrates to produce an unnatural novel triketide, 4-hydroxy-3,5-dimethyl-6-pentyl-pyran-2-one.

Chalcone synthase (CHS) (EC 2.3.1.74) is a plant-specific type III polyketide synthase (PKS) that catalyzes a stepwise condensation of the C_6-C_3 unit of 4-coumaroyl-CoA (**1a**) as a starter with three C_2 units from malonyl-CoA (**2**) (Scheme 1A).[1,2] The reaction is initiated by binding of the CoA-linked starter molecule to an active site cysteine. After three rounds of malonyl-CoA decarboxylation and polyketide chain extension reaction, regiospecific Claisen-type cyclization and aromatization of the enzyme-bound tetraketide intermediate leads to formation of 4,2′,4′,6′-tetrahydroxychalcone (naringenin chalcone) (**5a**) (Scheme 1A). In CHS enzyme reactions in vitro, a triketide and a tetraketide pyrone, bisnoryangonin (BNY) (**3a**)[3] and 4-coumaroyltriacetic acid lactone (CTAL) (**4a**)[4] are also obtained as early-released derailment byproducts. Recent crystallographic and structure-based mutagenesis studies on alfalfa (*Medicago sativa*) CHS2 and other CHS-superfamily enzymes revealed the structural details of the active site governing the polyketide formation reaction.[5]

In previous studies, we have demonstrated that CHS from *Scutellaria baicalensis* has remarkably broad substrate specificity toward either the starter molecule or the extension unit of the polyketide chain elongation reaction.[6,7] Thus,

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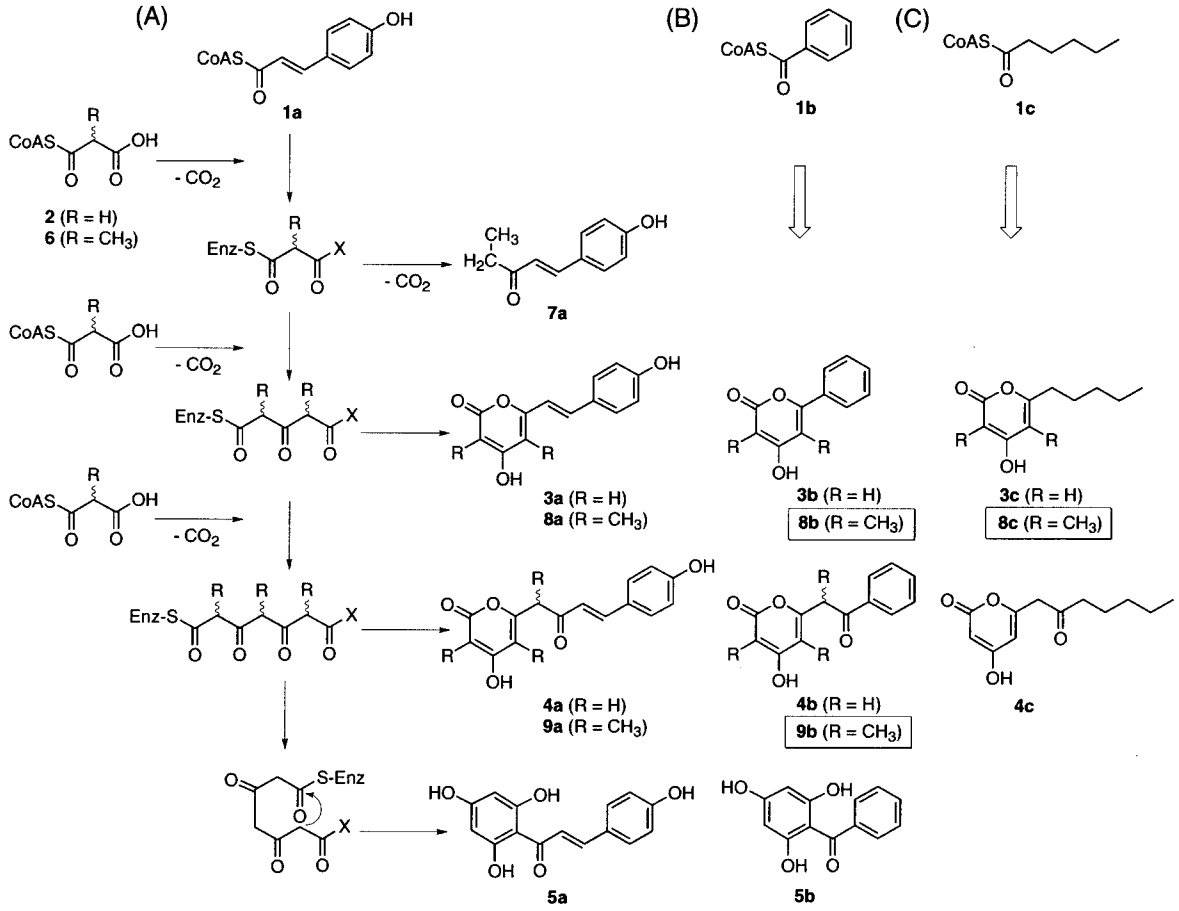
Scheme 1. Enzymatic Formation of Naringenin Chalcone (**5a**) from 4-Coumaroyl-CoA (**1a**) and Malonyl-CoA (**2**) and Formation of Unnatural Polyketides from Alternate Starter (**1b** and **1c**) and Nonphysiological Extension Substrates (**6**) by Recombinant *S. baicalensis* CHS



instead of 4-coumaroyl-CoA, the enzyme accepted a wide variety of starter molecules, both aromatic and aliphatic CoA esters of different chain length, including benzoyl-CoA (**1b**) and hexanoyl-CoA (**1c**), and efficiently performed sequential decarboxylative condensations with malonyl-CoA (**2**) to produce a series of chemically and structurally different unnatural polyketides (Scheme 1B and 1C).[6] On the other hand, instead of malonyl-CoA, the enzyme also accepted methylmalonyl-CoA (**6**) as an extension unit of the poyketide chain elongation reaction. When incubated with 4-coumaroyl-CoA (**1a**) and methylmalonyl-CoA (racemic) (**6**), *S. baicalensis* CHS afforded an unnatural C_6-C_5 aromatic diketide (**7a**) along with a BNY- and a CTAL-type pyrone byproduct, **8a** and **9a** (Scheme 1A).[7] The extraordinary broad substrate specificity of the plant PKS enabled us to develop a chemical library of unnatural novel polyketides. In this paper, in order to further examine the versatility of the catalytic function of the enzyme, we carried out enzymatic conversion experiments in which *both* the starter and the extension unit of the CHS enzyme reaction were *simultaneously* replaced with nonphysiological substrates.

When recombinant *S. baicalensis* CHS[8] was incubated with benzoyl-CoA (**1b**) as a starter and methylmalonyl-CoA (racemic) (**6**) as an extension substrate, two products were isolated by reverse-phase HPLC (Figure 1A).[9] As in the case of previously reported enzyme reactions with methylmalonyl-

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(8) Recombinant *S. baicalensis* CHS with an additional hexahistidine tag at the C-terminal was expressed in *E. coli* and purified by Ni-chelate affinity chromatography as described before. The K_M and k_{cat} values were 36.1 μM and 1.26 min^{-1}, respectively, for 4-coumaroyl-CoA and 22.9 μM and 0.83 min^{-1}, respectively, for malonyl-CoA.

(9) The reaction mixture contained 54 μM of benzoyl-CoA (or hexanoyl-CoA), 108 μM of methylmalonyl-CoA, and 20 μg of the purified recombinant *S. baicalensis* CHS in a final volume of 500 μL of 100 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA. Incubations were carried out at 30 °C for 18 h and stopped by addition of 50 μL of 20% HCl. The products were then extracted with 600 μL of ethyl acetate and separated by reverse-phase HPLC (column, TSK-gel ODS-80Ts, 4.6 × 150 mm, Tosoh Co., Ltd., Japan; flow rate, 0.8 mL/min; UV detection at 290 nm). The HPLC eluent for separation of **8b** and **9b** was 50% aqueous MeOH containing 0.05% TFA, while for separation of **8c**, gradient elution was performed with H_2O and MeOH containing 0.05% TFA (0−20 min, linear gradient from 55 to 75% MeOH; 20−30 min, linear gradient from 75 to 80% MeOH). For large-scale enzyme reactions, benzoyl-CoA (15.0 mg, 17.3 μmol) and methylmalonyl-CoA (30.0 mg, 34.6 μmol) were incubated with 52.6 mg of purified recombinant CHS in 300 mL of 100 mM phosphate buffer, pH 8.0, containing 1 mM EDTA at 30 °C for 18 h. On the other hand, hexanoyl-CoA (5.0 mg, 5.8 μmol) and methylmalonyl-CoA (10.0 mg, 11.5 μmol) were incubated with 20.0 mg of purified recombinant CHS in 100 mL of the buffer at 30 °C for 18 h. The enzyme still retained ca. 20% of activity after 18 h at 30 °C.

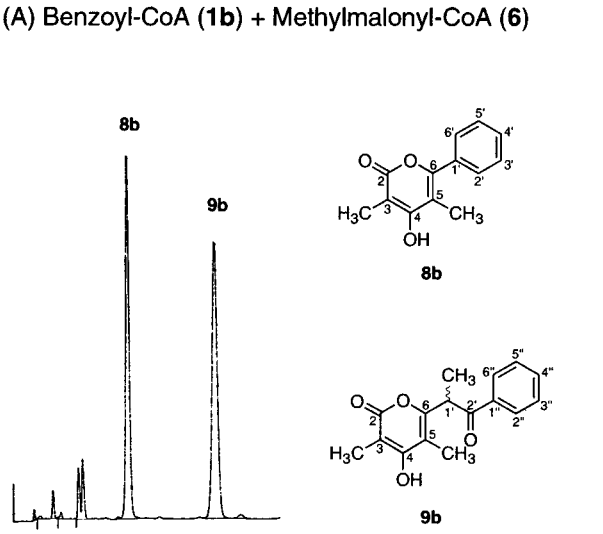

Figure 1. HPLC profile of the enzyme reaction products (A) from benzoyl-CoA (**1b**) and methylmalonyl-CoA (**6**) and (B) from hexanoyl-CoA (**1c**) and methylmalonyl-CoA (**6**). 4-Hydroxy-3,5-dimethyl-6-phenyl-pyran-2-one (**8b**) (R_t = 22.7 min), 4-hydroxy-3,5-dimethyl-6-(1-methyl-2-oxo-2-phenyl-ethyl)-pyran-2-one (**9b**) (R_t = 12.9 min), and 4-hydroxy-3,5-dimethyl-6-pentyl-pyran-2-one (**8c**) (R_t = 20.8 min).

CoA as a substrate, formation of the nonphysiological products was so slow (apparently at least 100 times slower than that of chalcone) that we carried out the incubation for 18 h.[7,9]

The two products showed UV spectra similar to those of BNY and CTAL, respectively, suggesting the structures of 4-hydroxy-3,5-dimethyl-6-phenyl-pyran-2-one (**8b**)[10] and 4-hydroxy-3,5-dimethyl-6-(1-methyl-2-oxo-2-phenyl-ethyl)-pyran-2-one (**9b**),[11] respectively (Scheme 1B). The LC-ESIMS spectrum of BNY-type **8b** gave a parent ion peak $[M + H]^+$ at *m*/*z* 217, while that of CTAL-type **9b** gave a parent ion peak at *m*/*z* 273, indicating that the polyketide chain elongation reaction had terminated after two or three condensations with the C_3 units from methylmalonyl-CoA. Further confirmation of the structures was obtained by NMR spectroscopic data of **8b** and **9b** isolated from a large-scale enzyme incubation (0.5 mg each, 7 and 6% yields from 15.0 mg of benzoyl-CoA, respectively).[9] The ^{1}H NMR spectrum of **8b** showed two methyl singlets (δ 2.04 and 1.96) of a α-pyrone ring, while that of **9b** revealed two methyl singlets (δ 2.05 and 1.84) and one methyl doublet (δ 1.46, J = 6.7 Hz) coupled with a proton (δ 4.85, J = 6.7 Hz), in addition to the benzene ring proton signals. Moreover, the homonuclear (^{1}H−^{1}H COSY) and heteronuclear (HMQC and HMBC) correlation spectra were uniquely consistent with the proposed structures of the unnatural novel methylated triketide **8b** and tetraketide **9b**.

On the other hand, when incubated with hexanoyl-CoA (**1c**) and methylmalonyl-CoA as substrates, *S. baicalensis* CHS afforded 4-hydroxy-3,5-dimethyl-6-pentyl-pyran-2-one (**8c**)[12] as a single product (Figure 1B).[9] The LC-ESIMS spectrum of **8c** gave a parent ion peak $[M + H]^+$ at *m*/*z* 211, indicating the polyketide chain elongation reaction had terminated after two condensations). The ^{1}H NMR spectrum of **8c** obtained from a large-scale enzyme reaction (0.5 mg each, 22% yield from 5.0 mg of hexanoyl-CoA)[9] showed two methyl singlets (δ 1.93 and 1.89) of an α-pyrone ring in addition to the aliphatic chain proton signals. Furthermore, the homonuclear (^{1}H−^{1}H COSY) and heteronuclear (HMQC and HMBC) correlation spectra conclusively supported the structure.

This is the first demonstration of the enzymatic formation of unnatural polyketides from alternate starter and nonphysiological extension substrates by a plant PKS. The enzyme accepted benzoyl-CoA and methylmalonyl-CoA as substrates and catalyzed the formation of a novel methylated triketide **8b** as well as a tetraketide pyrone **9b**[13] (Scheme 1B), while hexanoyl-CoA and methylmalonyl-CoA afforded a novel

(10) HPLC: R_t = 22.7 min. LC-ESIMS: *m*/*z* 217 $[M + H]^+$. UV: λ_{max} 232 and 310 nm. ^{1}H NMR (500 MHz, CD_3OD): δ 7.87 (2H, ddd, J = 8.2 Hz, 1.2 Hz, 1.2 Hz, H-2′ and H-6′), 7.46 (3H, m, H-3′, H-4′, and H-5′), 2.04 (3H, s, Me-5), 1.96 (3H, s, Me-3). ^{13}C NMR (125 MHz, CD_3OD): δ 170.2 (C-4), 168.6 (C-2), 156.0 (C-6), 134.5 (C-1′), 129.3 (C-4′), 128.5 (C-2′ and C-6′), 128.1 (C-3′ and C-5′), 111.4 (C-5), 98.2 (C-3), 10.6 (Me-5), 7.8 (Me-3). HRMS (FAB): found for $[C_{13}H_{12}O_3 + Na]^+$, 239.0669; calcd, 239.0684.

(11) HPLC: R_t = 12.9 min. LC-ESIMS: *m*/*z* 273 $[M + H]^+$. UV: λ_{max} 246 and 290 nm. ^{1}H NMR (500 MHz, CD_3OD): δ 7.87 (2H, ddd, J = 7.0 Hz, 1.0 Hz, 1.0 Hz, H-2″ and H-6″), 7.56 (1H, ddd, J = 7.4 Hz, 7.4 Hz, 1.0 Hz, H-4″), 7.45 (2H, ddd, J = 7.4 Hz, 7.0 Hz, 1.0 Hz, H-3″ and H-5″), 4.85 (1H, t, J = 6.7 Hz, H-1′), 2.05 (3H, s, Me-5), 1.84 (3H, s, Me-3), 1.46 (3H, d, J = 6.7 Hz, Me-1′). ^{13}C NMR (125 MHz, CD_3OD): δ 198.4 (C-2′), 167.4 (C-4), 167.4 (C-2), 157.6 (C-6), 137.0 (C-1″), 133.0 (C-4″), 128.6 (C-3″ and C-5″), 127.9 (C-2″ and C-6″), 109.8 (C-5), 98.0 (C-3), 43.8 (C-1′), 13.4 (Me-1′), 8.5 (Me-5), 7.5 (Me-3). HRMS (FAB): found for $[C_{16}H_{16}O_4 + Na]^+$, 295.0956; calcd, 295.0946.

(12) HPLC: R_t = 20.8 min. LC-ESIMS: *m*/*z* 211 $[M + H]^+$. UV: λ_{max} 290 nm. ^{1}H NMR (500 MHz, CD_3OD): δ 2.53 (t, 1H, J = 7.4 Hz, H-1′), 1.93 (s, 3H, Me-5), 1.89 (s, 3H, Me-3), 1.64 (m, 2H, H-2′), 1.34 (m, 4H, H-3′ and H-4′), 0.90 (t, 3H, J = 6.9 Hz, H-5′). ^{13}C NMR (125 MHz, CD_3OD): δ 172.9 (C-2), 167.8 (C-4), 159.4 (C-6), 109.3 (C-5), 98.6 (C-3), 31.0 (C-3′), 30.0 (C-1′), 26.5 (C-2′), 21.7 (C-4′), 12.7 (C-5′), 8.7 (Me-5), 7.5 (Me-3). HRMS (FAB): found for $[C_{12}H_{18}O_3 + Na]^+$, 233.1148; calcd, 233.1153.

(13) Tetraketide pyrone **9b** was obtained as a racemic compound. Utilization of chiral methylmalonyl-CoA as a substrate would provide useful information on the stereochemistry of the enzyme reaction.

methylated triketide pyrone **8c** (Scheme 1C).[14] The structures of the reaction products were apparently different from that of chalcone, the regular product of CHS. The extraordinary broad substrate specificity and the versatility of catalytic function of the recombinant *S. baicalensis* CHS were quite remarkable. The in vitro promiscuity of the enzyme seems to be a common feature of type III PKS enzymes of plant and bacterial origin.[1,2] Recently, it has been reported that RppA, a bacterial type III PKS that normally catalyzes formation of 1,3,6,8-tetrahydroxynaphthalene from five molecules of malonyl-CoA, also showed broad substrate specificity and accepted methylmalonyl-CoA and acetoacetyl-CoA to produce a C-methylated pyrone, 3,6-dimethyl-4-hydroxy-2-pyrone.[15] Furthermore, CHS2 from *Pinus strobes* has been reported to catalyze a single extension of a diketide intermediate mimic to a triketide pyrone using methylmalonyl-CoA.[16]

Interestingly, formation of neither diketides nor tetraketides with a new aromatic ring system was detected in the assay mixtures. In contrast, as we have reported in a previous paper, when 4-coumaroyl-CoA (**1a**), the normal starter substrate, was incubated with methylmalonyl-CoA, most of the polyketide chain elongation reactions were terminated at the diketide stage and afforded phenylpentanone (**7a**) as a major product (Scheme 1A).[7] Presumably, loading of the less bulky starter molecule of benzoyl-CoA or hexanoyl-CoA into the coumaroyl-binding pocket of the active site enabled further polyketide chain elongation reactions (Scheme 1B). In the case of the aliphatic hexanoyl-CoA, the chain extension was interrupted at the triketide stage and afforded the triketide pyrone as a single product. As mentioned above, due to the presence of the additional bulky methyl group of the extension unit analogue, the enzyme reactions with methylmalonyl-CoA were extremely slow compared with those with malonyl-CoA, the normal extension substrate. For further production of unnatural novel poyketides, manipulation of the plant PKS reactions by utilizing substrate analogues and rationally engineered mutant enzymes is now in progress in our laboratories.

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Supporting Information Available: Set of NMR spectra (^{1}H NMR, ^{1}H−^{1}H COSY, HMQC, and HMBC) of 4-hydroxy-3,5-dimethyl-6-phenyl-pyran-2-one (**8b**), 4-hydroxy-3,5-dimethyl-6-(1-methyl-2-oxo-2-phenyl-ethyl)-pyran-2-one (**9b**), and 4-hydroxy-3,5-dimethyl-6-pentyl-pyran-2-one (**8c**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Similar product pattern was observed for recombinant stilbene synthase (STS) from *Arachis hypogaea*, another plant type III PKS that normally catalyzes formation of resveratrol from 4-coumaroyl-CoA and malonyl-CoA. Furthermore, our preliminary results showed that both *S. baicalensis* CHS and *A. hypogaea* STS accepted cinnamoyl-CoA, phenylacetyl-CoA, and isovaleryl-CoA as a starter substrate to produce methylated BNY- and CTAL-type pyrones.

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